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Progressive Pigmentary Regression in Fishes Associated With Cave Environments.

PRISCILLA RASQUIN.
The American Museum of Natural History.

(Plate I; Text-figure 1).

INTRODUCTION.[1]

The Mexican blind characins which are derived from an eyed river form, *Astyanax mexicanus* (Filippi), are of several varieties. One of these, *Anoptichthys jordani* Hubbs and Innes, from La Cueva Chica, retains an optic capsule in various stages of degeneration and some degree of pigmentation, both of which evidently vary with the genetic constitution of the individual fish. The second variety, as yet undescribed, from Cueva de los Sabinos (see Alvarez, 1946) has a mere remnant of the optic capsule unconnected with the brain by any optic nerve and a pigmentary system which consists only of a few scattered melanophores (Breder, 1944). These chromatophores are so few in number that dispersion or concentration of their granules cannot bring about any noticeable difference in the coloration of the fish. The ancestral river form is characterized by large eyes and a considerable amount of black pigment. A dark stripe extends from behind the head to the tail, ending in a definite black spot just anterior to the caudal fin.

The pigmentation of these three forms showed a different quantitative reaction to conditions of light and darkness. The river fish conformed to the general pattern of chromatophore function, assuming a light phase when kept in darkness and an extremely dark phase when blinded and kept in light. The fish from La Cueva Chica kept in darkness were noticeably more pink in color and more translucent than those kept in light. On the other hand the Sabinos fish retained their translucent pinkish condition regardless of light conditions and in addition presented a yellowish appearance. This study was undertaken to determine what agents were responsible for these differences in coloration.

Most of the blind fishes studied in this report were brought from the Mexican caves by Mr. Benjamin J. Dontzin who was in charge of the expedition to Mexico sent out from the Department of Fishes and Aquatic Biology of the American Museum of Natural History in the spring of 1946.

The earlier part of the experimental work was carried out in the laboratories of the Department of Animal Behavior. The later portions were carried out in a laboratory of the Department of Birds which through the kindness of Dr. R. C. Murphy has been turned over to our use for blind fish studies.

MATERIALS AND METHODS.

Three kinds of fish were used. Of these, the *Astyanax mexicanus* were aquarium-raised descendants of fish collected from the Rio Tampaon, San Luis Potosi, Mexico, at Pujal, near La Cueva Chica in 1940. These will be referred to as "river fish." The second kind was *Anoptichthys jordani*, called "Chica fish." Some of these used were members of an eleventh generation raised in the light and some were brought directly from La Cueva Chica, at Pujal in the same Mexican state. Normal specimens were studied together with some in which the optic nerve remnants had been severed and some in which the blind capsule as a whole had been removed.[2] Not only were forms raised in the light observed, but animals kept in complete darkness, both operated and unoperated for a period of nine months, were used. The eleventh generation fish at the time of operation were no more than two months of age. The ages of the fish collected in Mexico are unknown. The third kind, as yet undescribed, were specimens brought from Cueva de los Sabinos, north of Valles, Mexico. Most of these were recent arrivals in the laboratory but specimens were available which had been kept under ordinary laboratory light conditions for more than two years. Those which were studied as having been in darkness, had traveled from Mexico in tightly closed tins painted on the inside with black asphaltum paint and were exposed to light for only five days before being placed in dark, light-tight tanks. Others were specimens killed and fixed in the cave without ever having been subjected to light other than the momentary hand lamps of the collectors. The river fish which were maintained in darkness had been put into the dark tank at the age of one month and maintained under such con-

[1] An abstract of this paper appeared under the same title (Rasquin, 1946).

[2] These fish are the same individuals which had been used by Breder and Rasquin (1947) for other studies.

ditions for more than two years. The only light to which they were exposed was that of a 10 watt ruby bulb used at feeding time.

One hundred Chica fish were operated on August 12, 1945. Fifty had both optic nerve remnants severed, and of these 25 were placed in a dark tank and 25 in a tank in which the ordinary conditions of daylight obtained. The second 50 had the entire blind capsule removed and were split 25 and 25 in the same manner. Fifty more fish which served as controls were subjected to the same process of anesthesia and distributed between similar dark and light tanks. Anesthesia was accomplished by immersing the fish in a 1 per cent. solution of urethane made with the water from the tanks in which the fish had been living. The river fish had the optic nerves severed in the same way and the fact that they were completely blinded was verified by their immediate assumption of a permanent dark phase, and a wandering continuous swimming activity characteristic of the cave varieties. The operations on the normally blind Chica fish were considered necessary for they had been previously proved to be light sensitive through the intermediation of the blind capsule (Breder and Gresser, 1941 a and b).

Denervation of melanophores in the caudal fin was accomplished by the technique used by Fries (1942), that is, a cut was made in the caudal fin at right angles to the fin rays, causing a degeneration of the nerve fibers distal to the cut. Four fish were used for this part of the experiment, one river fish, one Sabinos, one normal Chica and one Chica whose blind capsules had been removed. The experiments were repeated. One per cent. urethane was used in the first trial but anesthesia was found to be unnecessary and was abandoned in the second trial.

Serial sections were made of the heads of the experimentally blinded fish to check the severing of the optic nerves in those fish which were subjected to this method of blinding. Whole mounts of skin and peritoneum fixed in formalin as well as fresh preparations were studied for melanophores. For the demonstration of fat, formalin fixed tissue was treated with 2 per cent. osmic acid. A strip of skin, scales and underlying muscle tissue was removed extending from the dorsal to the ventral mid-line just anterior to the dorsal fin. These results were checked with frozen sections stained with Sudan IV.

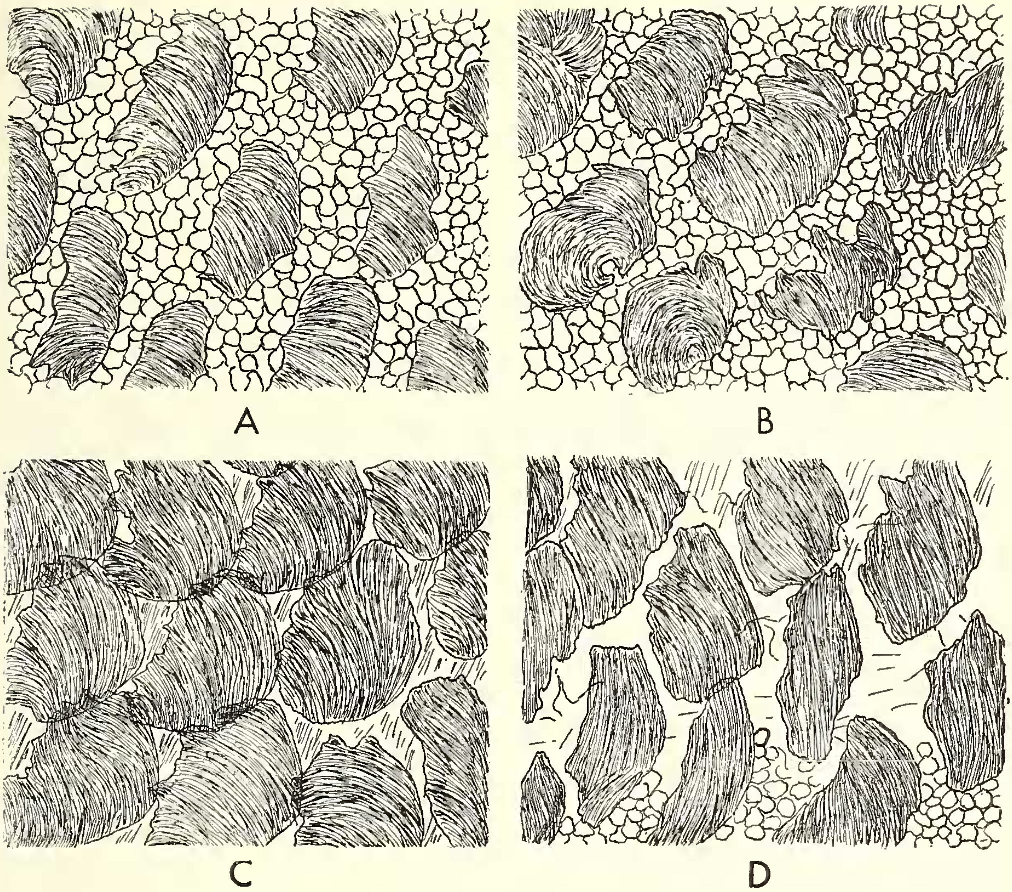
Drawings of the distribution of the guanin were made free hand using the dissecting microscope at magnification of approximately 20 diameters. In each case the left side of the fish is pictured at a region just posterior to the body cavity and below the mid-line where the differences in guanin deposition are most marked and most easily recognizable.

The Chica fish subjected to light and darkness were kept in 6 tanks, 3 in light and 3 in darkness, so arranged that the water was kept circulating through all tanks by means of a pump. The same amounts of food were given to each tank. No plants were used in the light tanks. The water became green due to growth of natant algae but this plant material was circulated through all tanks by the action of the pump. See Breder and Rasquin (1947) for specific details of this apparatus.

Experimental Results.

The river fish under normal light conditions exhibits a heavy pigmentation throughout the body. Sections show a deep layer of melanophores in the dermis and another less concentrated layer immediately beneath the surface epithelium. Layers of melanophores are seen in the fascia between the myomeres of the body musculature. Pigment cells are numerous along the pathway of the blood vessels, particularly along the aorta and its branches all the way down the body to the caudal fin. There is a heavy concentration in the meninges covering the brain and spinal cord. Internal pigmentation varies in individual fish but is always heavy. Large melanophores almost always in the dispersed condition are found in the peritoneum. These cells are characterized by broad blunt branches which resemble a syncytium forming an almost complete layer of pigment protecting the abdominal cavity. Examination of a river fish which had been kept in total darkness for a period of two years revealed a much different picture, agreeing in general with the observations of Ogneff (1908, 1911) on goldfish, Murisier (1920-21) on trout and Odiorne (1937) on catfish. Much of the pigmentation was lost following the long sojourn in the dark. The superficial pattern was preserved, the lateral stripe was still distinct but the fish appeared to be in a very light phase due not only to concentration of the granules within the cells but to considerable reduction in the number of pigment cells. The internal pigmentation showed this reduction more clearly than the superficial pigmentation. The aorta and its branches were still marked by a few melanophores but the pigmentation surrounding the branches did not extend nearly as far distally as in the fishes raised in the light. Melanophores in the peritoneum were sparse and scattered and the syncytial appearance was not nearly so pronounced.

Examination of Chica fish killed and fixed in the cave, and never subjected to living conditions in the light, show varying conditions of pigmentation, in general those fish having better differentiated eyes having more pigment. However, this is not always the case. Occasionally heavily pigmented fish with completely blind capsules are seen and these are in a permanently dark phase corresponding to river fish which have been operatively blinded, but such specimens are



TEXT-FIG. 1. Semi-diagrammatic sketches of the fat and guanin deposits in the blind fishes. Sketches show the left side of the fishes at a region just posterior to the body cavity and below the mid-line at a magnification of approximately 20 diameters. **A.** Sabinos fish in the light. **B.** Sabinos fish in the dark. **C.** Chica fish in the light. **D.** Chica fish in the dark.

relatively rare (Breder, 1942). In general in those fish with eyes, melanophores are seen in the skin and the lateral stripe is usually evident. Melanophores can be seen along the blood vessels in the fascia between the myomeres of the dorsal region as well as in the meninges of brain and spinal cord. Those fish with a completely blind capsule showed much less pigment, little being seen along the blood vessels. All had melanophores in the skin and peritoneum and in fixed specimens conditions of both dispersal and concentration of granules in these cells were seen side by side. This would seem to indicate that the cells have not lost any power to react.

After removal of the blind capsules from these Chica fish or after severing of the remnants of the optic nerves rendering them insensitive to light, they did not enter upon a dark phase nor in the period of nine months that they were kept in the light did they produce a noticeable darkening in pigmentation such as did the blinded river fish. Between those fish raised in the dark and those raised in the light, whether operated upon or not, there was not a sufficient difference in the melanin content to account for the difference in translucence and coloration. The pinkish color of the dark raised fish is due to the color of the blood which shows through the skin. In those Chica fish with a considerable amount of pigment some of this color is undoubtedly blocked off by the position of melanophores about the blood vessels and in those raised in the dark the absence of the heavily pigmented layer of the peritoneum permits the viscera to be seen. Microscopic examination of live Chica fish brought into the light reveals concentrated melanophores, while those living in the light and subjected to the same amount of handling showed melanophores in a more dispersed condition.

In the Sabinos fish, which are insensitive to light, the superficial pigmentation consists of a few scattered melanophores in the skin and there is no concentration of pigment cells forming a lateral stripe. In six fish examined which had been kept in the light for more than two years no melanophores were found between the myomeres. A few were seen scattered along the dorsal aorta and a few were present in the peritoneum and meningeal tissues. Microscopic examination of one of these fishes alive and in the light showed the melanophores to be in the punctate and stellate conditions; full expansion was not observed.

As a result of this observation it was thought best to determine whether the innervation of the melanophores in the Sabinos fish was also in a process of degeneration. Living in the light for two years produced no gross visible increase in pigmentation, nor was any such increase produced in the Chica fish through the rearing of eleven generations in the light. Accordingly four fish were subjected to cuts of two or three rays in the caudal fin, causing a degeneration of nerve fibers distal to the cut (Fries, 1942). One river fish was used, one Sabinos and two Chicas. Of the two Chicas one was an old fish and the other was a younger fish which had had the optic capsule removed several months previously. A darker streak was observed within five minutes on the tail of the river fish, indicating expansion of the melanophores when released from innervation by the cutting of the nerve. The others could not be seen to have such an immediate reaction, probably because of the fewer pigment cells present. Handling incident to inspection under the microscope caused a dropping off of the part of the fin distal to the cut. The cuts were therefore repeated on the other half of the fin and the fish returned to the aquaria to await microscopic examination after some healing had been accomplished. Care was taken to make the observations within the time limit before regeneration began (Abramowitz, 1935). Eleven days after the operations the fins were examined under the microscope. All melanophores in the denervated areas were in a dispersed condition in all three types of fishes, although the Sabinos showed more cells in the stellate condition than in a state of full expansion.

A freshly killed Sabinos fish which had been in the light only five days was examined. This fish was of unknown age, brought from the cave in Mexico. The melanophores were mainly contracted, but occasional ones were seen in the stellate condition and some in the head region were fully expanded, possibly incident to death. Death obviously did not cause immediate dispersion of all the melanophores for the majority were concentrated. No pigment cells were present in the peritoneum or mesentery.

Thirty Sabinos fish which had been killed and fixed in the cave without ever being subjected to conditions of light were examined microscopically for pigment cells. In none of these specimens were any melanophores present in the peritoneum. The meninges showed a few scattered pigment cells. The melanophores of the skin were scattered and very few in number, and all were contracted. One fish examined after living in the light for six months still showed no melanophores in the peritoneum or mesenteries, but the skin and the meninges covering the brain showed a definite increase in the numbers of these cells. In the six fishes examined which had been living in the light for more than two years, the melanophores were found in all the places which are characteristic for fishes, including the peritoneum. The appearance of these peritoneal melanophores is particularly noticeable because there were none present before subjection to light but the actual increase in numbers is small.

Xanthophores contribute somewhat to the coloration of all these fishes, being numerous in the caudal and adipose fins. The anal fins contain scattered xanthophores which deepen in shade from a lemon yellow to almost a brick red in some cases, as they approach the anterior border of this fin. The deeply colored cells are more frequently noted in the river fish, both male and female, and are less frequently found in the Chica and Sabinos types.They are not always present in the river fish. Scattered xanthophores are seen in the dorsal fin and on the head and dorsal body regions. In the river fish the presence of many melanophores obscures the yellow color of the other chromatophores.

The Sabinos fish gives a distinctly yellow impression, particularly the male, but it cannot be said that this is due to any greater number of xanthophores as compared with the Chica fish or the river fish. A consideration of these three forms raised in the light shows a steady progression in the quantity of fat deposition. The river fish has very little, the Chica fish show a conspicuous amount and the Sabinos fish show great quantities of fat not only in the abdominal cavity but laid down in layers between the dermis and the musculature. It is this yellow fat showing through the skin which gives the Sabinos fish its yellowish appearance. The nature of and reason for this fat deposition is not within the province of this paper but will be made the subject of a later communication.

A variation in the amount of guanin crystals seems to be chief cause of the difference in coloration in the blind fishes. The river fish under the dissecting microscope presents a body covered with a sheath of guanin. The crystals are laid down on each scale, in the dermis underlying the scales and in the tissue between the peritoneum and the body wall so that a view of the internal organs or of the color of the blood is completely blocked. The abundant pigmentation which overlies the guanin prevents much of the silvering from showing through. In the Chica fish raised in the light the same guanin deposition holds true. Scales are well marked and the guanin is deposited between scales so that the fish becomes an opaque silver, brilliantly flashing in the light. The lack of a dense overlying layer of melanin makes these fish much more conspicuous against any natural background than the river fish. In the dark-raised specimens, however, guanin is not nearly so abundant. Crystals are found laid down heavily on the scales and intervening tissue along the sides of the fish, particularly along the lateral pigmented stripe, but in the scales ventral and dorsal to this line guanin is seen on the scales alone, and is sparse and somewhat erratically laid down. This permits the color of the inner tissues to show through the skin. Some guanin crystals are seen between the peritoneum and body wall of these dark-raised fish but are not nearly as abundant as in the light-raised Chicas or in the river fish. This increased opacity due to guanin deposition takes place within three weeks after exposure to daylight conditions and occurs in the Chica fish whether or not they were made operatively insensitive to light. Therefore the optic remnants which have been shown to make these fish light sensitive cannot be involved in this reaction to light.

The Sabinos fish, whether kept in light or darkness, have less guanin deposits than the Chica fish raised in the dark. If there is an increase in guanin in these fishes when exposed to light it is not great enough to cause any change in coloration, nor to be detected by inspection under the dissecting microscope. Specimens newly brought from the cave showed no differences from those which had been kept in the light in the laboratory for more than two years. Guanin is seen on the scales alone and is not abundant. The colors of blood, viscera and fat are clearly visible through the relatively transparent skin.

Discussion.

The blind cave derivatives of the Mexican characin *Astyanax mexicanus* offer interesting material for the study of pigmentation since they have lived for generations in an environment totally without light. They show a progressive loss of pigmentation which in general is correlated with the stages of eye degeneration. It seems probable that the stock from La Cueva Chica is contaminated by the occasional entry of eyed fish from the river since some of the fish, particularly from the pool nearest the river, have functional eyes and normal distribution of melanin. The others from pools further removed from the river are mainly blind and lack much of the melanin which characterizes the eyed form. The fish from Cueva de los Sabinos on the contrary show no evidence of such contamination with river forms and are completely blind and indifferent to light. Pigmentation is still further reduced, melanin is present only in scattered melanophores in the skin and few are seen internally.

In the river fish the distribution of internal pigment agrees in general with that described by Gordon (1931) for *Platypoecilus* and with what seems to be normal for typical fishes. All the layers mentioned by Weidenreich (1912) are represented, that is epidermal and dermal melanin, together with perineural, perivascular and coelomic pigmentation. The peritoneum is particularly characterized by expanded chromatophores which seem to form a syncytium. Whether or not these cells are actually anastomosed cannot be determined by ordinary histological methods. It is not surprising to find these cells in a dispersed condition since Gilson (1926) observed that the melanophores of the peritoneum of *Fundulus* rarely showed a contracted form regardless of the condition of the superficial chromatophores. Ginsburg (1929) studied the living melanophores in embryos of *Lebistes reticulatus,* some of which would ultimately be drawn internally with the yolk sac and claimed that the melanophores could become anastomosed and the granules wander from one cell to another. Shanes and Nigrelli (1941), using a technique involving polarized light, seemed to find some evidence to support the hypothesis that these cells are interconnected. However, river fish which originally had this heavy pigmentation picture in the peritoneum lose it entirely when kept for some time in darkness and what melanophores are left are separated and show little evidence of anastomosed branches.

Sumner and Wells (1933) noted a loss of melanophores from the peritoneum of *Lebistes reticulatus* which had been adapted to a white background. Such optic stimuli as altered background conditions played no part in the reaction shown by the river fish kept in darkness. They were raised in light tight tanks from the age of one month to more than two years, receiving no retinal images in that time save that of the 10 watt carbon ruby dark room bulb used only at feeding time for checking the conditions of the tank. Coonfield's work (1940) on *Pomacentrus leucostictus* has more bearing on this problem for he found that the melanophores of eyed or blinded embryos kept in total darkness were contracted even more than those kept over white backgrounds.

Melanophores in the cave fishes of both varieties are pale and seem to have few granules although the cells are clearly outlined and there is no difficulty in deciding whether or not the granules are in a dispersed condition. Goodrich (1927) in a study of *Oryzias latipes* found that the red and white varieties had as many melanophores as the brown, the only difference being in the amount of melanin within the cells. This is not the case here, for the blind cave fishes when compared with the ancestral river form are seen to have lost great quantities of melanophores. The loss of color is not due to fewer granules within existing cells but to the actual loss of great numbers of pigment cells.

The pigmentation of the phenotypic variants shown by the Chica fish differs so widely that it is impossible to tell what reaction has been effected by light or darkness on the numbers of melanophores. Rendering these fish insensitive to light by removal of the blind capsule causes no difference in their pigmentary reactions to light. In those with little pigmentation there are so few melanophores in the skin that mere dispersal of the granules would have no gross visible effect on the coloration and an increase in pigmentation could only be effected by a tremendous increase in the numbers of melanophores. The reaction of light probably does cause a slight increase in numbers since both the river fish and the less pigmented Sabinos fish show such an increase and the innervation of the pigmentary system has been proved to be functioning in all three types of fishes. Denervation experiments have shown that the power to react has not been lost in the cave fishes. Such an increase is more easily seen in the Sabinos fish because of the presence of even fewer pigment cells than in the Chica fish and because of the appearance of these cells in places where they were not observed before subjection to light. According to Osborn (1940, 1941) the melanophores must be in an expanded condition to produce an increase in melanin in existing cells and to produce an increase in the numbers of melanophores.

The Sabinos fish represent a stage of still further degeneration as regards pigmentation and eyes (Breder, 1944). The optic capsule as a structure is gone and there is no connection to the brain by an optic nerve. The increase in the numbers of melanophores which occurs when these fish are kept for long periods in the light is not sufficiently great to cause any visible difference in the coloration. The time required for this slight increase is in excess of six months. Fishes removed from darkness or brought out from the cave show little internal pigmentation, none at all in the peritoneum and only a few scattered contracted cells over the dorsal region of the skin. Microscopic examination of fish kept more than two years in the light shows a definite increase in numbers in the meninges and perivascular tissues, melanophores have appeared in the peritoneum and the dorsal skin surface appears lightly peppered with pigment cells. These are not heavily laden with granules and the cells seem to be in the stellate condition rather than fully contracted or expanded.

No such magnitude of reaction takes place in the cave fish comparable to that which Odiorne (1937) demonstrated with blinded catfish. Osborn (1941) also demonstrated the apperance of large numbers of melanophores on the ventral surfaces of flounders by subjecting them to strong lighting from below through a glass bottomed tank. Both these forms are genetically provided with large numbers of melanophores and they may possess a potential for increase under proper conditions of stimulation which is evidently greater than that inherent in the blind fish. Melanophores in the Sabinos fish, which have appeared as a response to light, are not sufficiently great in number to be apparent macroscopically. Microscopic examination, however, shows them to be arranged in a differential pattern comparable to that of the river fish or to the general distribution which usually obtains in fishes which live in conditions where the lighting is from above. The increase in numbers is greatest over the dorsal region and gradually decreases ventrally. Before any response to light occurs, the few melanophores found in the Sabinos fish are seen over the same region, while none are seen below the midline. The Chica fish kept in darkness also show the same differential.

As far as could be determined, life in the cave environments has produced no such loss of xanthophores in the fishes as is the case with melanophores. These yellow chromatophores appear in about the same quantities in all three varieties of fishes, being obscured in the river fish by the greater numbers of melanophores. In the Sabinos fish kept either in light or darkness the xanthophores far outnumber the melanophores.

Several investigators have observed (Meyer, 1931; Kuntz, 1917; Murisier, 1920-21; Sumner and Wells, 1933) that conditions of light which favor a decrease in melanin content favor an increase in guanin and guanophores. This is at variance with observations on the cave fishes. Chica fish when raised in darkness and brought into ordinary daylight conditions deposit enough guanin in three weeks' time to change them from a rather translucent, pinkish color to an opaque silver. This reaction takes place whether or not the fish are rendered operatively insensitive to light and therefore cannot be mediated through any optical nerve connection with the brain.

Craig-Bennett (1931) has observed that the blue color of the iris of the breeding male stickleback, *Gasterosteus aculeatus* Linnaeus, is due to the reduction in the amount of guanin in the guanophores, and a pinkish color of the operculum which precedes the development of erythrophores is due to the color of the blood in the gills which shows through after the guanin has been reduced. He found that the color changes would take place whether the male fish were kept in light or darkness. In the Chica fish the added accumulation of guanin takes place regardless of the sex of the fish. River fish which had lived in total darkness more than two years, including one specimen which had evidently been spawned in the tank, showed no less guanin than those which had been raised and kept in the light. The Sabinos fish showed no more guanin when kept in the light for more than two years than those which were kept in the dark or those which were received fresh from the cave environment.

Two conditions obtain in the Sabinos fish which make them distinctive in coloration from the Chica fish. One of these is the lack of a deposition of guanin in response to exposure to light and the other is the presence of an abundant deposition of subcutaneous fat which is independent of the action of light. The color of the blood and fat renders the Sabinos fish a distinct yellowish-pink as compared with the silvery Chica type. It is possible that there is some fundamental physiological difference between these two forms, but whether or not the lack of guanin and the presence of fat are interrelated in some physiological process is not clear.

Summary.

1. A series of Mexican characins, ranging from a fully eyed form (*Astyanax mexicanus*, living in rivers) to a blind light-negative form (*Anoptichthys jordani* from La Cueva Chica) and a blind light-indifferent form (*Anoptichthys* sp. from Cueva de los Sabinos), exhibits gross color differences which are referable to reduction in melanin and guanin and increase in fat deposition in the sequence above listed.
2. The pinkish coloration of both blind forms kept in darkness, which is due to the blood showing through the skin, is masked in the river form by abundant melanin found in skin and peritoneum and about the large blood vessels and nerves.
3. When kept in light the "Chica" form deposits an almost complete investment of guanin effectively masking the pink coloration but the "Sabinos" form remains pink since it shows no such reaction to light.
4. Melanin and guanin obscure the small amount of river fish fat, but a progressive increase in fat deposition is evidenced by the more yellowish color of the "Sabinos" fish as compared with the translucent "Chica" form kept in darkness.
5. Melanophores in all three varieties of fish have lost no power of dispersion and concentration of granules and exposure to light results in a definite increase in numbers of melanophores in the river fish and in the "Sabinos" form.
6. No change was found in numbers of xanthophores, the yellow color of the caudal fins of the cave forms being due to the relative absence of melanophores which obscure the xanthophores in the river fish.
7. Increase in guanin and melanin is a direct effect of light since it occurs whether or not the fish are rendered optically indifferent to light.

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EXPLANATION OF THE PLATE.

PLATE I.

Photomicrographs of whole mounts of the peritonea of the river fish and the Sabinos fish showing the condition of the melanophores after subjection to light and darkness. Magnification 110 ×.

Fig. 1. River fish in the light.

Fig. 2. River fish in the dark.

Fig. 3. Sabinos fish in the light.

Fig. 4. Sabinos fish in the dark. American Museum of Natural History photographs.